Universidade de Lisboa

Faculdade de Medicina Dentária

Insights on the chemical ageing of Acrylic Reline Resins

Fábia Aurélia Vieira Alexandre

Dissertação

Mestrado Integrado em Medicina Dentária

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Dissertação orientada pela Professora Doutora Maria Cristina Bettencourt Neves e coorientada pela Professora Doutora Ana Francisca Bettencourt

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Resumo

As resinas acrílicas de rebasamento autopolimerizáveis são frequentemente usadas para reajuste de próteses dentárias aos tecidos subjacentes, após a progressiva reabsorção do osso alveolar. Garantem desta forma uma melhor retenção, suporte e estabilidade das próteses removíveis.

Contudo, sabe-se que a resina acrílica da base da prótese pode atuar como reservatório de microrganismos, tendo assim o potencial de favorecer a formação de biofilmes.

A estomatite protética associada a infeção por Candida é uma forma de candidíase oral, comum em pacientes idosos reabilitados com próteses dentárias removíveis, tendo uma prevalência de 45-70%.

Apesar de vários organismos contribuírem para o desenvolvimento desta doença, o fungo Candida *albicans* é o principal agente causal. Esta é uma condição de origem multifatorial, pois está associada a outros fatores locais e sistémicos, como a utilização de próteses mal adaptadas, higiene protética insuficiente, utilização contínua da prótese sem períodos de descanso, secreção salivar reduzida devido a medicação ou radioterapia, carências nutricionais, utilização de antibióticos de largo espetro, entre outros fatores. Sabe-se que nesta situação, o pH salivar tende a ser mais ácido.

Deste modo, a inibição da formação de biofilmes de C. *albicans* é particularmente importante na prevenção da estomatite protética.

A clorexidina é um agente microbiano com largo espectro de ação contra microrganismos, entre os quais a C. *albicans*, sendo prescrita usualmente em Medicina Dentária como solução de bochecho a 0,2%. No entanto, a maioria do agente é removido da cavidade oral durante a primeira hora pela reposição salivar, diminuindo as suas propriedades terapêuticas.

Por esta razão, têm sido investigadas alternativas para o tratamento da estomatite protética, entre as quais a incorporação de clorexidina nas resinas acrílicas. A libertação deste fármaco a partir do biomaterial, ocorre a um ritmo constante, inibindo a aderência de microrganismos, com riscos mínimos de toxicidade sistémica. No entanto, alguns

estudos apontam que esta incorporação pode afetar as propriedades físicas das resinas acrílicas, ao contribuir para um aumento da porosidade.

Ao veicular o fármaco em resinas acrílicas de rebasamento é importante referir que estas apresentam, com o tempo, algumas características poucos satisfatórias, como a abrasão, porosidade e alteração de cor.

Sabe-se que a cor é uma das mais importantes propriedades clínicas dos materiais dentários. O sistema CIELab é o mais utilizado para a medição instrumental da cor, pois permite estudar a diferença de cor (ΔE). Num outro sistema, denominado CIELch descrevem-se as cores com o mesmo espaço de cor do sistema CIELab, mas através de coordenadas cilíndricas. A conversão dos valores entre estes dois sistemas é meramente matemática, sendo o valor de L igual em ambos.

O principal objetivo deste estudo foi avaliar o efeito do envelhecimento químico, através da alteração de pH, na libertação de clorexidina de resinas acrílicas de rebasamento e avaliar a estabilidade da cor destes biomateriais.

Três materiais foram selecionados para avaliação no presente estudo: Kooliner, Ufi Gel Hard (materiais para rebasamento direto) e Probase Cold (material para rebasamento indireto). Para cada material foram produzidos três grupos de espécimes, sendo um de controlo (sem clorexidina) e dois com incorporação de clorexidina em duas concentrações: 1% e 2,5% (m/m). Estes espécimes foram submetidos a três diferentes protocolos de envelhecimento químico: a) sempre em saliva artificial a pH 7; b) ciclos de 6h em pH 5 alternados com 18h em pH 7; c) ciclos de 6h em pH 3 alternados com 18h em pH 7. Foram avaliados um total de 135 espécimes em forma de cilindro (com cerca de 12mm de comprimento e 6mm de diâmetro). De modo a estudar a libertação da clorexidina, os cilindros foram armazenados individualmente em frascos graduados de 5mL e cobertos por saliva, num rácio de 1g/5mL. Estes foram posteriormente incubados a 37ºC e, em intervalos de tempo específicos (20 min,1, 6, 24,30, 48, 78, 96, 168, 186, 240, 264, 336, 378, 504, 522, 600, 672 horas), foram pipetados 450µL a partir de cada frasco para uma placa de micropoços. As amostras foram de seguida analisadas num espectrofotómetro a 255nm e as absorvâncias foram convertidas em concentrações. Nos mesmos intervalos de tempo, 450µL de saliva artificial foram renovados em cada frasco, de modo a simular a constante renovação salivar.

A medição da cor foi realizada antes e após o estudo de libertação, com recurso a um espetrofotómetro (*EasyShade Vita*). Os valores de *l*, *c* e *h* foram convertidos para o sistema CIELab e a diferença de cor foi calculada (ΔE). De seguida, este valor foi transformado em unidades *NBS (National Bureau of Standards)*, para indicar a diferença de cor numa perspetiva clínica.

Os resultados foram analisados estatisticamente através de testes não paramétricos, pelo método de *Kruskal-Wallis*, seguindo-se múltiplas comparações pelos testes de *Mann-Whitney*, com correção de *Bonferroni*. Foi considerado um nível de significância igual a 5%.

Os resultados demonstraram que para todos os grupos em estudo, uma elevada libertação inicial foi seguida por uma libertação mais lenta e controlada, a qual permaneceu durante todo o tempo do estudo, o que vai de encontro a outros estudos. Em relação à alteração de pH, verificou-se que em saliva artificial pH 3 e 7, ocorreu maior libertação de clorexidina. Relativamente ao efeito das diferentes composições dos materiais na libertação da clorexidina, o Kooliner apresentou maior libertação em pH 3 e 7, no entanto, em pH 5 e 7, não se observaram diferenças significativas entre o Kooliner e o Ufi Gel. O Probase Cold, por sua vez, apresentou sempre a menor libertação de clorexidina.

No que diz respeito ao efeito da diferente percentagem de incorporação de clorexidina na libertação da mesma, nos grupos em que se realizou alteração de pH, não se verificaram diferenças entre os grupos de 1% e 2,5%.

No presente estudo, a libertação cumulativa máxima foi de apenas 1,48%, o que significa que apenas uma pequena porção da clorexidina inicialmente incorporada, foi libertada para a saliva artificial. Este resultado poderá dever-se ao facto dos espécimes serem de dimensões reduzidas. No entanto, após comparação das concentrações cumulativas de clorexidina libertada com a respectiva concentração mínima inibitória (MIC), verificou-se que, todos os grupos em estudo, apresentaram concentrações superiores a esses valores da MIC. Assim, os resultados sugerem que 1% de clorexidina é o suficiente para inibir C. *albicans*, o que reduz o risco do desenvolvimento de reações alérgicas pelo hospedeiro, dado se tratar de uma pequena concentração.

Relativamente à avaliação da estabilidade da cor, todos os espécimes apresentaram valores de $\Delta E>0$, o que significa que em todos se verificou uma alteração

de cor. Não se verificaram diferenças significativas em relação aos meios com diferentes pHs, no entanto, a cor parece ter sido afetada pela diferente composição dos materiais. O Kooliner registou a maior alteração de cor, seguido pelo Probase Cold e pelo Ufi Gel Hard. Observou-se ainda que a incorporação de clorexidina, promoveu maior diferença de cor, no entanto, não se observaram diferenças entre 1% e 2,5%.

Relativamente a limitações do estudo, os espécimes não reproduzem a superfície protética, pelo que futuros estudos deverão ter este aspeto em conta, bem como são necessários estudos microbiológicos e de biocompatibilidade. Em relação ao pH, é necessário padronizar os procedimentos experimentais, por forma a comparar com outros estudos. Para além disso, é necessário ter em conta que a alteração de cor das resinas acrílicas pode ser afetada por diversos fatores.

Tendo em conta a libertação de clorexidina e a alteração de cor, o Ufi Gel Hard pode ser uma escolha eficiente em situações agudas de estomatite protética, podendo ser depois substituído por Probase Cold, de modo a manter a libertação do fármaco e prevenir recidivas.

Palavras-Chave: resinas acrílicas de rebasamento; Estomatite Protética; Clorexidina; Envelhecimento químico; alteração de cor

Abstract

The main purpose of this study was to evaluate the effect of chemical ageing on the chlorhexidine release and color stability of three acrylic reline resins – Kooliner, Probase Cold and Ufi Gel Hard, loaded with chlorhexidine.

For each material, control group and chlorhexidine 1% and 2.5% (w/w) experimental groups (n=5) were produced. They were placed in artificial saliva and submitted to three different protocols of chemical ageing: a) at pH 7; b) cycles of 6h at pH 5 interchanching with 18h at pH 7; c) cycles of 6h at pH 3 interchanching with 18h at pH 7. A total of 135 specimens were evaluated in this study. The saliva samples were analyzed by UV spectroscopy and chlorhexidine content was determined. Color measurements were performed before and after the release study, using a spectrophotometer. The values registred were converted to CieLab system, and the overall color change **(ΔE)** was calculated and converted to *NBS (National Bureau of Standards)* units, to denote the color differences in a clinical perspective.

Data were submitted to Kruskall-Wallis tests ($p<0.05$), followed by multiple comparisons by Mann-Whitney test with Bonferroni correction.

When subjected to acid conditions, all acrylic reline resins had a higher drug release. At pH 5 and 7, the pattern of release of Kooliner and Ufi Gel Hard was similar and Probase Cold revealed the lowest amounts of chlorhexidine released. Results of color stability demonstrated that all samples showed a color alteration. There were no differences between the three release media, but it was affected by different acrylic reline resins composition. Kooliner showed the highest color change, followed by Probase Cold and Ufi Gel Hard. Chlorhexidine incorporation leaded to a higher color change.

Keywords: Acrylic reline resins; Denture stomatitis; Chlorhexidine; Chemical ageing; Color change

1. Introduction

Autopolymerizing acrylic reline resins are frequently used in dentures readjustment to the continuous reabsorbed underlying tissues, to provide better retention, support and stability of removable prostheses (Marra *et al*. 2012; Neves *et al.* 2013; Mendes de Oliveira *et al*. 2014).

However, it has been shown that denture base acrylic resins may act as reservoirs for microorganisms and have the potential to support biofilm formation (Marra *et al*, 2012; Pero *et al*. 2013)

Candida-associated denture stomatitis is a common form of oral candidosis, associated to elderly denture wearers, with a prevalence of 45–70% (Salim, Moore, *et al*. 2012a). It manifests as diffuse inflammation of the denture-bearing areas, and provides multiple challenges for its management. (Ryalat *et al*. 2011).

Although this disease is a mixed multispecies candidal–bacterial biofilm infection, C. *albicans* is known as the principal causative agent (Redding *et al*. 2009; Rautemaa and Ramage 2011; Ryalat *et al*. 2011; Salim, Moore, *et al.* 2012a). However, it has a multifactorial etiology, since it is associated with other local and systemic factors, such as, acid salivary pH or reduced saliva secretion, poor denture hygiene, continuous denture wear, a long-term antibiotic therapy, hormonal therapy, nutritional deficiency, among others (Monroy *et al*. 2005; Redding *et al*. 2009; Rautemaa and Ramage 2011).

Monroy and colleagues in 2005, reported atrophic denture stomatitis in 50 patients and the pH average in saliva of them was of 5.2.

Therefore, inhibition of the formation of C. *albicans* biofilms is very important in preventing the development of denture stomatitis (Redding *et al*. 2009).

Chlorhexidine (CHX) is a positively-charged molecule that binds to the negatively-charged sites on the candidal cell wall; it destabilizes the cell wall and interferes with osmosis. (Salim, Moore, *et al.* 2013a). This antifungal is presented in many oral delivery formulations, but it is used primarily as 0.2% mouthwash with a topical mode of action. In this case, most of the agent is removed from the oral cavity during the first hour due to the diluent effect of saliva and the cleansing effect of the oral

musculature, possibly reducing its therapeutic efficacy (Ryalat *et al.* 2011; Salim, Moore, *et al.* 2013a).

Several attempts to incorporate antifungal agents or antiseptics into tissue conditioners and denture acrylic resins have been reported, to avoid the formation of biofilm on denture base resin surfaces. A sustained release delivery system for treatment of denture stomatitis using CHX incorporated into a tissue conditioner has been investigated (Riggs *et al*. 2000; Ryalat *et al*. 2011; Marra *et al.* 2012; Salim, Silikas, *et al*. 2012b; Salim, Moore, *et al*. 2013a; Salim, Silikas, *et al*. 2013b; Bertolini *et al.* 2014). Direct delivery of the drug to the site of infection reduces the risk of systemic side effects or drug–drug interactions (Salim, Moore, *et al*. 2012a; Salim, Silikas, *et al*. 2013b;). Chlorhexidine incorporation into denture acrylic resins has shown better results than other drugs, like fluconazole, on releasing and microbiological tests (Amin *et al.* 2009; Redding *et al*. 2009; Salim, Moore, *et al*. 2012a; Salim, Silikas, *et al*. 2013b).

It appears that the physical properties of the resin were affected due to the incorporation of the drug particles, which may dissolve and result in porosity in the acrylic base (Amin *et al.* 2009; Ryalat *et al.* 2011; Salim, Silikas, *et al*. 2013b; Sousa, 2014).

Also, besides their many advantages, such as, ease manipulation, low cost, adequate physical and mechanical properties, biocompatibility, and satisfactory appearance, acrylic reline resins exhibit, unsatisfactory characteristics over time, such as loss of elasticity, abrasion, porosity, and color change (Waldemarim *et al*. 2013; Goiato *et al*. 2014;).

Color stability is one of the most important clinical properties for dental materials (Goiato *et al*. 2014).

In 1976, the International Commission on Illumination (Commission Internationale de l'Eclairage – CIE) developed a 3-dimensional color space based on axis "L" ("black"(0) to "white"(100) values); "a" ("green"(negative) to "red"(positive) values) and "b" (blue to yellow values) called **CIELab** system, which covers all colors visible to the human eye and allows studies in color difference in dental materials. The amount of color change may be described by the ΔE, which shows the distance between two given colors in this space. This scale has been used in several studies of acrylic resin

color stability in contact with pigmented liquids (Waldemarim *et al* 2013; Goiato *et al.* 2014), and is the mostly used system in color measurements (Stevenson *et al.* 2010).

Another system, known as **CieLch**, describes the color of the same system CIELab color space, but using cylindrical coordinates. The *L* expresses the lightness of the sample from black (0) and white (100), *c* is a measure of chroma saturation and represents the distance from the neutral axis, and *h* is a measure of hue and is represented by an angle between 0 ° and 360º. The conversion of CIELab values for CieLch is mathematical. The L values are the same in both systems (Vichi *et al*. 2004; Goiato *et al.* 2014).

Several instruments, such as colorimeters, spectrophotometers, spectroradiometers, and digital cameras, are widely used in color measurements in dentistry. Spectrophotometric measurements have been proved to outperform visual assessments in both reliability and reproducibility and they are the gold standard of color measuring devices. However, it has been demonstrated that both spectrophotometric and colorimetric devices can suffer edge loss effects, which happen when a device with a small window measuring a transparent material results in light scattering to the edge of the sample without being detected (Vichi *et al*.2004; Ren *et al*. 2015)

According to individual ability of the human eye to appreciate differences in color, three different intervals were used to distinguish the changes in color values, that is, $\Delta E<1$, imperceptible by the human eye; $1.0 < \Delta E < 3.3$, appreciated only by skilled person, clinically acceptable; $\Delta E > 3.3$, easily observed, these color changes are not clinically acceptable (Vichi *et al*.2004; Moon *et al*. 2015).

2. Objectives

The main purpose of this study was to evaluate the effect of chemical ageing on Chlorhexidine (CHX) release and the color stability of three acrylic reline resins, according to the following hypotheses:

1. H0: pH changes don't affect the drug release of the reline resins.
   H1: pH changes affect the drug release of the reline resins.

2. H0: pH changes don't affect the color stability of the reline resins
   H1: pH changes affect the color stability of the reline resins

3. H0: Materials differences don't affect the color stability of reline resins
   H1: Materials differences affect the color stability of reline resins

4. H0: CHX incorporation doesn't affect the color stability of the reline resins.
   H1: CHX incorporation affects the color stability of the reline resins.

3. Materials and Methods

In the present study, three auto-polymerizing acrylic resins (Table 3.1), presented in a liquid-power form, were selected because of their differences in terms of chemical composition. Two of these resins are direct reline resins: **Kooliner** (GC America Inc, Alsip, Illinois, USA) (Figure 3.1a) a non-crosslinking material, and **UfiGel Hard** (Voco GmbH, Cuxhaven, Germany) (Figure 3.1b), a cross linking material composed of pre-polymerized poly(ethyl methacrylate) (PEMA) powder particles and the monomers isobutylmethacrylate (IBMA) or 1,6-hexanodioldimethacrylate (1,6-HDMA), respectively. The other resin is an indirect reline resin, **Probase Cold** (Ivoclar Vivadent AG, Liechtenstein) (Figure 3.1c), a poly(methyl methacrylate) (PMMA) based material which has methylmethacrylate (MMA) as the monomer (Arima *et al*. 1995).

Table 3.1 - Materials under evaluation in the study.

Product	Manufacturer	Batch Number	P/L ratio (g/mL)	Composition	Curing Cycle
Kooliner (K)	GC America Inc., Alsip, Illinois, USA	1406232 (P) 1404241 (L)	1.4/1	P: PEMA L: IBMA	10 minutes 37ºC
Ufi Gel Hard (U)	Voco GmbH, Cuxhaven, Germany	1544083 (P) 1552273 (L)	1.77/1	P: PEMA L: HDMA	7 minutes 37ºC
Probase Cold (PC)	Ivoclar Vivadent AG, Liechtenstein	S41038 (P) U03356 (L)	1.5/1	P: PMMA L: MMA	15 minutes 40ºC 2-4 bar

P-Powder; L-Liquid; PEMA - polyethyl methacrylate ; IBMA - isobutyl methacrylate; HDMA - hexanediol dimethacrylate; PMMA - polymethyl methacrylate; MMA - methyl methacrylate

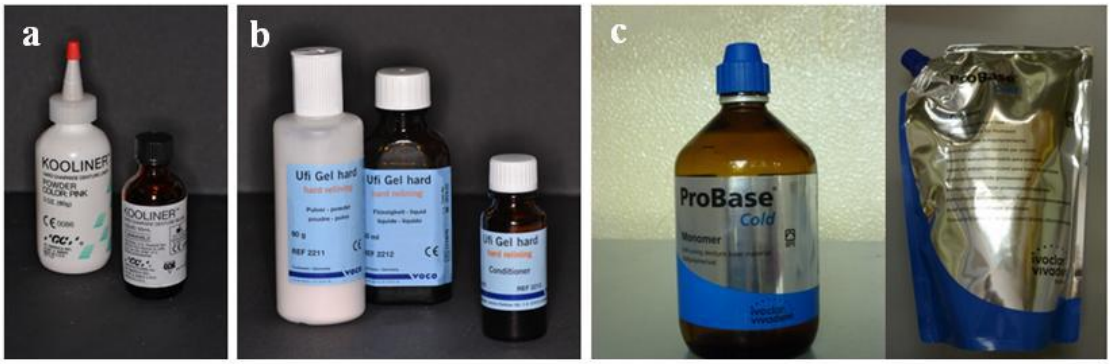


Figure 3.1 - Materials used in the study: a) Kooliner; b) Ufi Gel Hard; c) Probase Cold

3.1. Preparation of the specimens

The acrylic resins were manipulated according to the manufacturer's instructions (Table 3.1). The powder was weighted using a precision balance (Mettler Toledo) and the liquid was measured using a graduated pipette. On the experimental specimens, chlorhexidine diacetate monohydrate, (Panreac Applichem, Darmstadt, Germany) (CHX) (Figure 3.2a) at a proportion of 1% and 2.5% (w/w), was incorporated and mixed using a mortar and pestle for homogenization (Figure 3.2b).

The cylinder-shaped specimens (on average with 12 mm height and 6 mm diameter) (Figure 3.2c) were prepared using stainless steel molds (Figure 3.2d). For each material three groups of five specimens (n=5) were produced (one control group without CHX and two experimental groups with the CHX percentages mentioned), resulting in forty five specimens per material. (Scheme 3.1). A total of 135 specimens were prepared for this study.

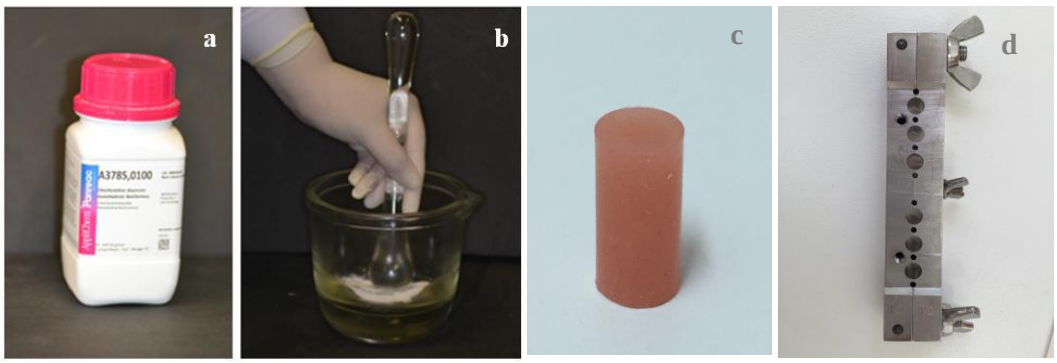


Figure 3.2 – Preparation of the specimens: a) Chlorhexidine diacetate monohydrate; b) Incorporation and homogenization; c) Cylinder-shaped specimen; d) Cylinder-shaped mold

In each preparation of Kooliner or Ufi Gel specimens, the materials dough was poured into the cylinder-shaped molds, maintained under compression at 37±2ºC, during the recommended polymerization time, (Table 3.1) and sealed with appropriate screws, in order to simulate the intraoral polymerization of direct reline resins. Polimerization of Probase Cold specimens was carried out in a pressure device (Ivomat, Ivoclar Vivadent, Liechenstein) (Figure 3.3) at recommended time, temperature and pressure (Table 3.1).

Figure 3.3 - Ivomat pressure device.

3.2. Analytical methodology

3.2.1. Standard stock and releasing solutions

A standard stock solution of 1000 μg/mL was prepared by dissolving 10 mg of CHX into 10 mL of deionized water. This solution was kept out of light, at room temperature. On each new measurement of CHX, a series of dilutions of the standard stock solution were prepared (125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95, 0.98 μg/mL).

The releasing solution used in the present study was artificial saliva at pH=7, at pH=5 and at pH=3, in order to understand how CHX would be released in the oral cavity. It was prepared according to a Faculty of Pharmacy University of Lisbon formula, courtesy of PhD student Joana Marto:

1) Boiling 50 mL (F12-ED Refrigerated/Heating Circulator) of phosphate buffer pH=7.0 (Anhydride disodium phosphate, Monosodium phosphate anhydride and Deionized water) at 60oC. Then sprinkled 0.05g of Xanthan gum into boiling buffer and stirring until total of xanthan gum was dissolved.

2) Dissolving 0.04g of Calcium chloride dihydrat (EW-N/EG-N balance), 0.08g of Sodium chloride and 0.08g of Potassium chloride in solution 1 and stirring until total of materials were dissolved.
3) Dissolving 15 g of Propylene glycol in solution 2 and stirring until total of Propylene glycol was dissolved.
4) Pouring the solution 3 into a graduated beaker and complete the solution with phosphate buffer pH=7.0 to 100 mL.
5) Adjusting the pH (Crison micro pH 2001) (Figure 3.4) of artificial saliva to 5 and 3 with HCl 1N.

These solutions were also kept out of light, at room temperature.

Figure 3.4 – Ph meter – Crison micro pH 2001.

3.2.2. Analytical technique

To measure the absorbance of each solution, a microplate reader (FLUOstar Omega – BMG LABTECH) (Figure 3.5) was used. Absorbance values were obtained using an Ultraviolet-Visible Absorbance Spectra detection mode, with a wavelength of 255nm, as recommended by other authors (Anusavice *et al.* 2006; Shen *et al*. 2010). The measurements were performed at 25ºC.

The CHX release concentrations were determined based on a linear calibration methodology, after subtracting the average of controls absorbance, at the corresponding time interval.

Figure 3.5 – Microplate reader

3.3. In vitro release studies

A preliminary study was performed to optimized further experimental protocols.

To study the release of CHX, the specimens were stored individually in graduated falcon tubes of 5mL and covered with artificial saliva, with a ratio of 1g/5mL (Figure 3.6a). The cylinders were assigned to 3 protocols of ageing in order to simulate oral conditions (A- immersion in artificial saliva at pH=7; B- cycles of 6h in artificial saliva at pH = 5 interchanging with 18h at pH = 7; C- cycles of 6h in artificial saliva at pH = 3 interchanging with 18h at pH=7). Between each change the specimens were washed with distilled water and dried with absorbent paper. Therefore, all the 9 experimental groups (n=5), for each material, were submitted to an ageing period of 28 days (Scheme 3.1).

The falcons were placed into an incubator at 37ºC (Memmert), with constant gentle shaking (300 rpm) (Figure 3.6b). At specific time intervals (20 min, 1, 6, 24, 30, 48, 78, 96, 168, 186, 240, 264, 336, 378, 504, 522, 576, 600, 672 hours) (Scheme 3.1), and after the falcons were agitated in a mixer (VELP Scientifica, Vortex), 450µL were pipetted from each falcon to a polystyrene flat-bottom microplate wells (96-well microplates) (150µL were pipetted to each well). At the same time intervals, 450µL of artificial saliva were replaced in each falcon, to simulate the constant salivary renovation. The samples were analyzed as previously described.

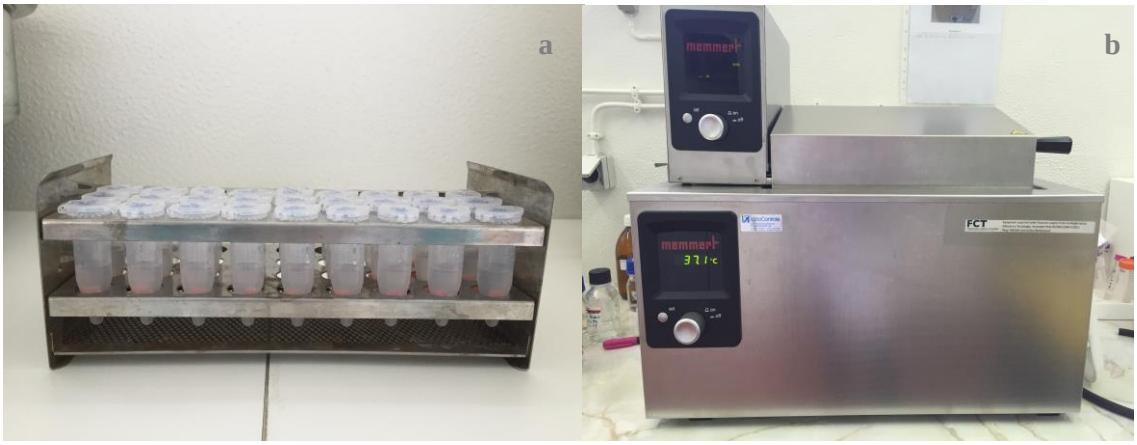


Figure 3.6 – Incubation of the specimens: a) in graduated falcon tubes with artificial saliva; b) at 37ºC, under constant gentle shaking by an incubator.

3.4. Color Measurement

Color measurements were performed using a spectrophotometer - *Easyshade Vita* (Figure 3.7a). The acrylic resin specimens and the reader were placed into a dark chamber (Figure 3.7b), so that the specimens were not exposed to light.

The initial color was measured by placing the specimen at the center of the reader. Both ends of the specimens were assessed and three measurements for each were made. The **l** (Lightness), **c** (chroma) and **h** (hue) values were registered and an average value was calculated and converted to **CIE Lab** system. $\boldsymbol{c}=(a^2+b^2)^{1/2}$ and $\boldsymbol{h}=\text{tang}^{-1}(b/a)$. L values were equal in both systems (Vichi *et al*. 2011; Goiato *et al.* 2014).

After that, each specimen was immersed in the designated test solution. After 28 days, the specimens were removed from the artificial saliva, rinsed with running water, and spot dried with tissue paper. The second color measurement was performed using the immersed specimens under the same conditions as before.

The overall color change $\boldsymbol{(\Delta E= [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2})}$ was calculated and converted to *NBS (National Bureau of Standards)* units, using the formula **NBS units=ΔE* x 0.92,** to denote the color differences in a clinical perspective (Pero *et al*. 2013; Goiato *et al.* 2014; Moon *et al*. 2015; Sousa *et al.* 2015).

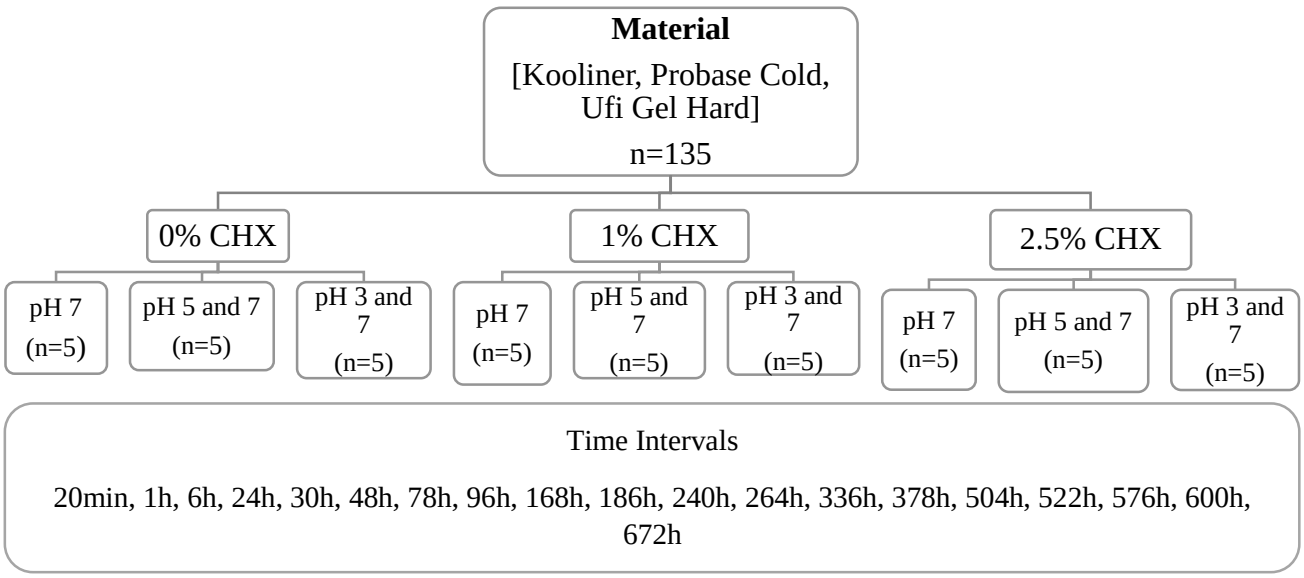
Figure 3.7 – Color Measurement of the specimens: a) Spectrophotometer *EasyShade Vita;* b) Dark chamber

3.5. Statistical Analysis

Descriptive statistics was carried out. Data were statistically analyzed using **SPSS Statistics 20** (SPSS Inc., Chicago, IL, USA).

Since data did not follow a normal distribution (verified by a Kolmogorov-Smirnov normality test), the results were submitted to nonparametric tests according to Kruskall-Wallis method, followed by multiple comparisons using Mann-Whitney tests with Bonferroni correction to determine whether there were specific significant differences among the variables under study.

In all statistical tests, it was considered the 5% level of significance (p<0.05).



Scheme 3.1 – Distribution of the specimens in the present study.

4. Results

4.1. CHX quantification

Linear relationship between absorbance peak areas at 255nm via a microplate reader UV- Visible Spectrophotometer and the CHX concentrations was established for each release solution. The analytical method showed good linearity in the 3 evaluated media (Figure 4.1).

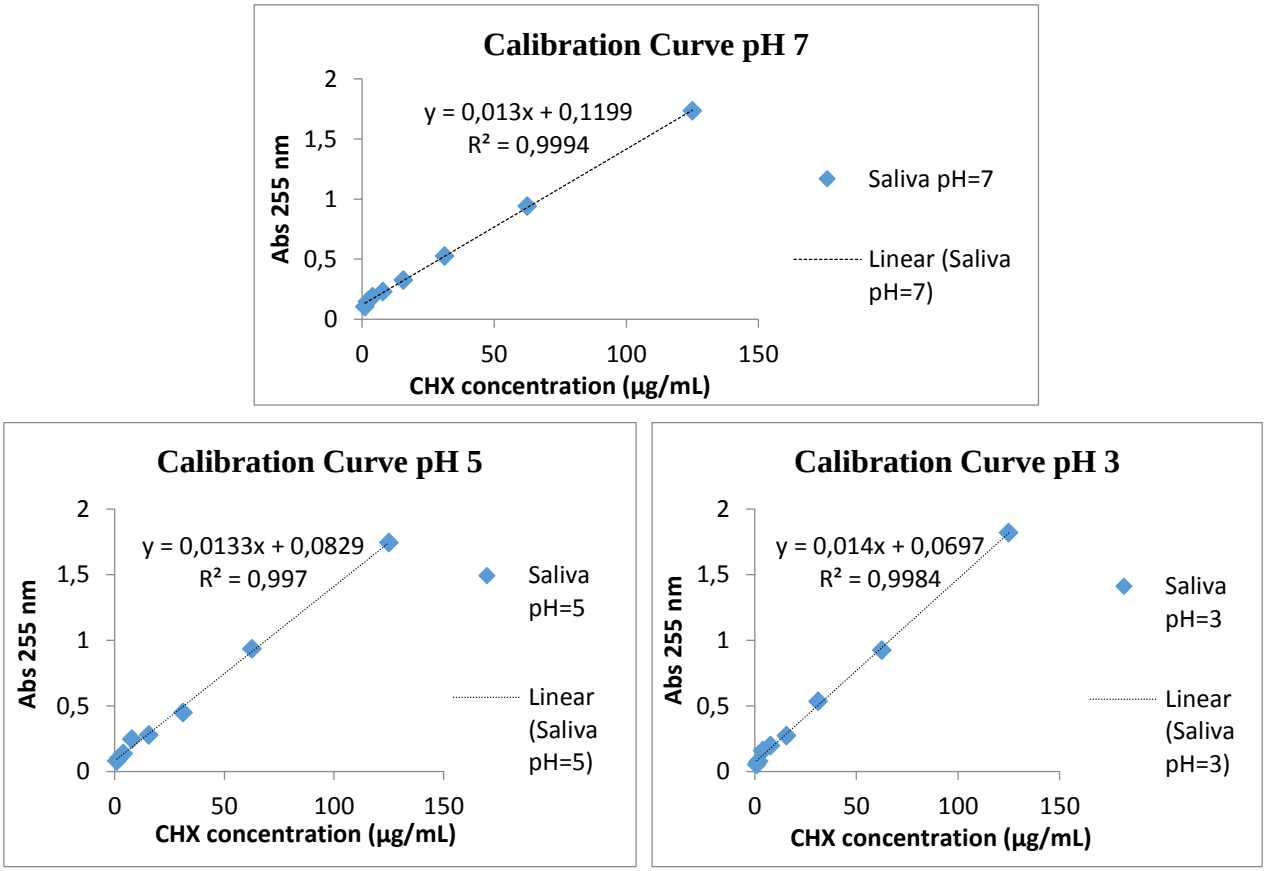


Figure 4.1 – Linear relationship between absorbance peak areas and the CHX concentrations for each pH used in the study: a) Saliva at pH 7; b) Saliva at pH 5; c) Saliva at pH 3

4.2. The effect of chemical ageing on the drug release

Specimens of three different materials - Kooliner (K), Ufi Gel Hard (U) and Probase Cold (PC), were evaluated in the present study, with CHX 1% and 2.5% (w/w), at pH 7, at pH 5 and 7, and at pH 3 and 7.

The results of CHX release for each group under study are showed below.

For CHX 1% (w/w) at pH 7, 11.11 µg/mL from K, 8.06 µg/mL from PC and 11.11 µg/mL from U were released until 48h of incubation. After 48h, PC showed a slower rate release than K and U (Figure 4.2).

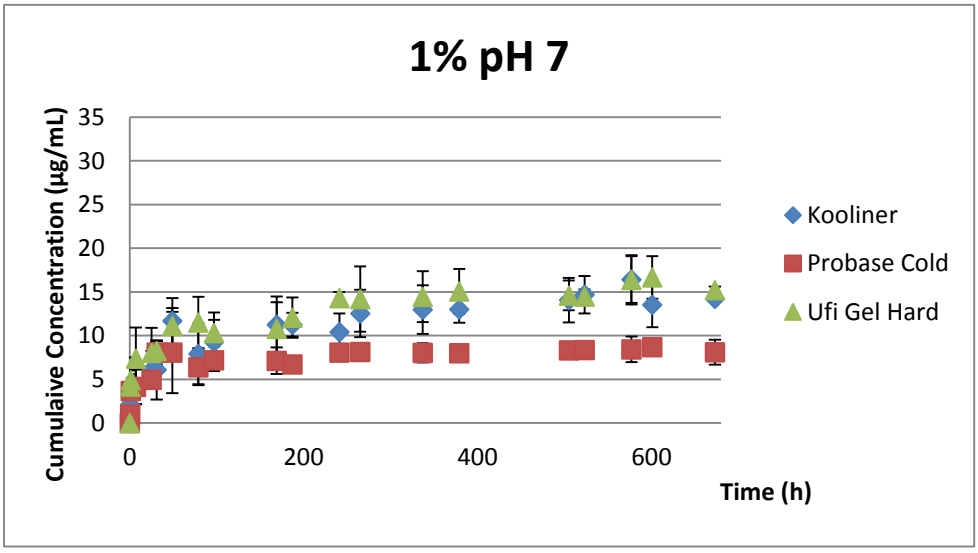


Figure 4.2 - Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 1% (w/w) at pH 7.

For CHX 1% (w/w) at pH 5 and 7, 12.85 µg/mL from K, 5.18 µg/mL from P and 10.07 µg/mL from U were released until 48h of incubation (Figure 4.3). After that, it shows a higher release than pH 7 (Figure 4.2), mainly for K and U.

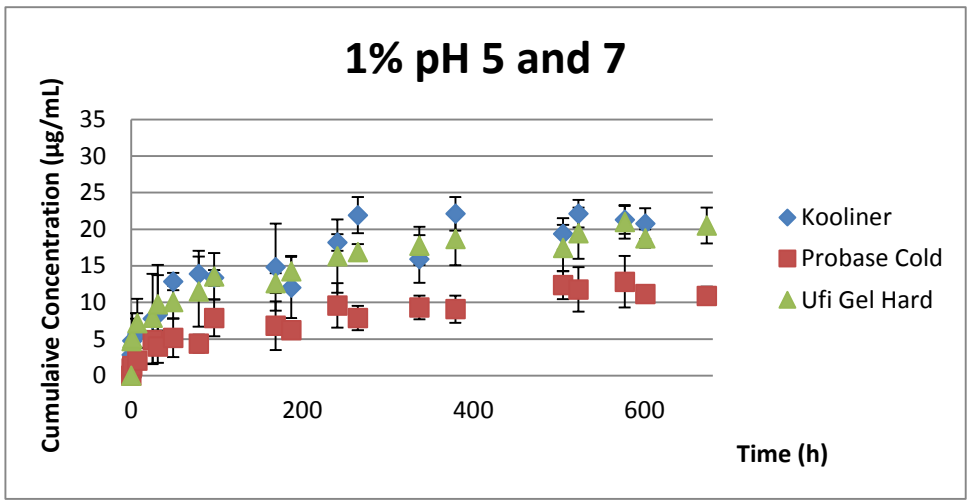


Figure 4.3 - Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 1% (w/w) at pH 5 and 7.

For CHX 1% (w/w) at pH 3 and 7, 12.00 µg/mL from K, 10.59 µg/mL from PC and 9.55 µg/mL from U were released until 48h (Figure 4.4). After that, it can be observed that, all the materials, show a higher rate of release than the other two media conditions showed above (Figure 4.2 and 4.3).

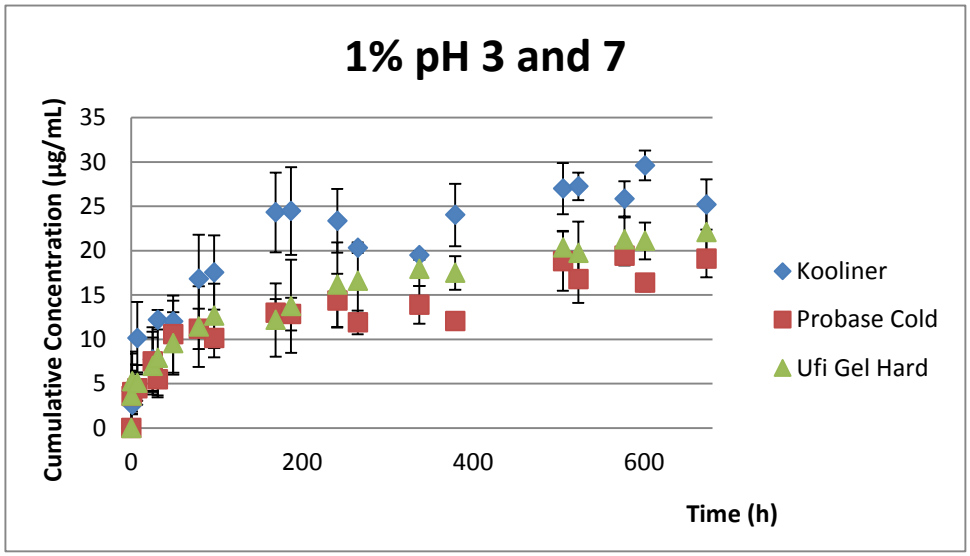


Figure 4.4 - Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 1% (w/w) at pH 3 and 7.

For CHX 2.5% (w/w) at pH 7, 14.77 µg/mL from K, 10.10 µg/mL from P and 12.77 µg/mL from U were released until 48h of incubation (Figure 4.5). There is a higher release than those specimens with CHX 1% (w/w) at the same pH (Figure 4.2).

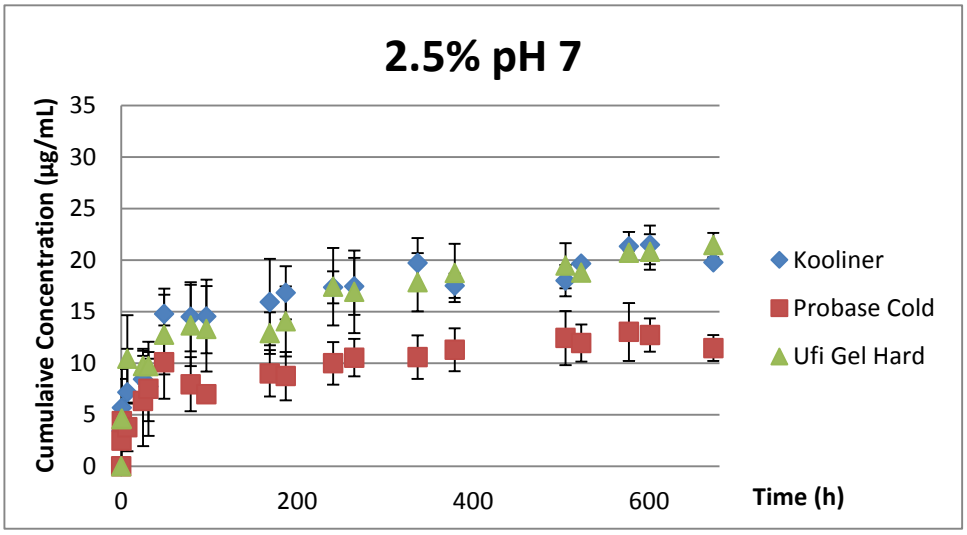


Figure 4.5 - Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 2.5% (w/w) at pH 7.

For CHX 2.5% (w/w) at pH 5 and 7, 11.98 µg/mL from K, 7.41 µg/mL from PC and 11.08 µg/mL from U were released until 48h. The release profile of the three materials was very similar (Figure 4.6) and there was a higher release than pH 7 (Figure 4.5).

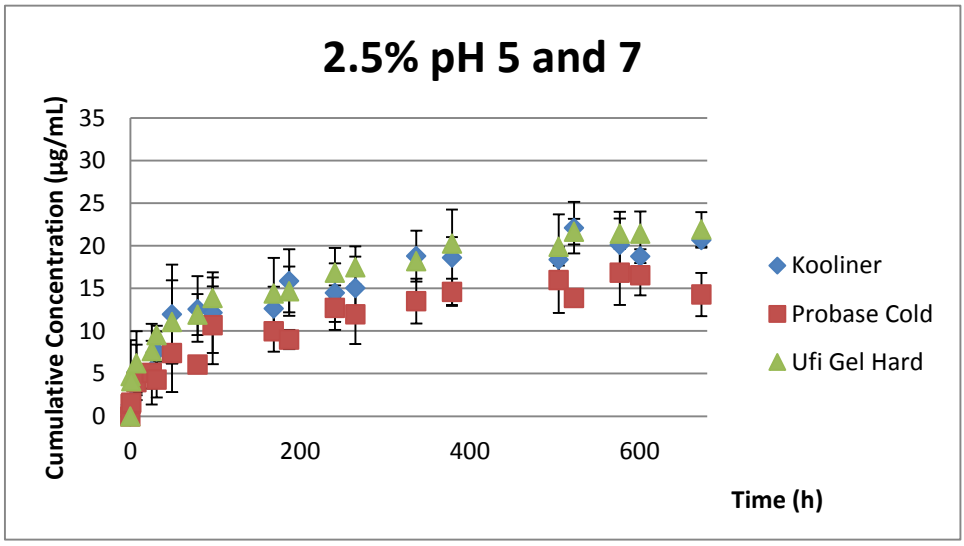


Figure 4.6 - Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 2.5% (w/w) at pH 5 and 7.

For CHX 2.5% (w/w) at pH 3 and 7, 10.74 µg/mL from K, 12.32 µg/mL from PC and 12.45 were released until 48h and (Figure 4.7). The release was higher than pH 5 and 7 (Figure 4.6).

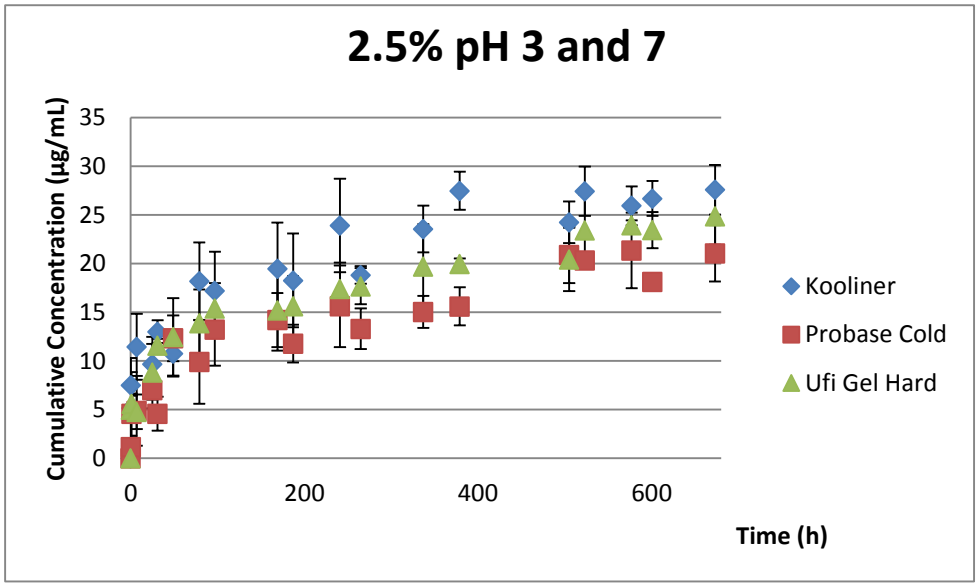


Figure 4.7 - Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 2.5% (w/w) at pH 3 and 7.

For all the evaluated acrylic reline resins, a high rate of initial release was followed by a slower and steadier release. The greatest amount of CHX release occurred within the first 48h of incubation, since more than 50% was released at this time.

The maximum cumulative release from the three materials for each group under study, is shown in Table 4.1. In all specimens, only a small amount of initial loaded CHX is release to the artificial saliva.

Table 4.1. Maximum concentration release for each group under study. Results are presented as M± SD.

Material	Group	Maximum Cumulative Release		
		(µg/mL)	% (w/w)	**t (h)**
Kooliner	1% pH 7	16.43± 2.15	0.82 ± 0.16	576
	1% pH 5 and 7	22.14 ± 2.13	1.11 ± 0.12	522
	1% pH 3 and 7	29.61 ± 1.66	1.48 ± 0.07	600
	2.5% pH 7	21.48 ± 1.41	0.43 ± 0.08	600
	2.5% pH 5 and 7	22.12± 3.01	0.45 ± 0.13	522
	2.5% 3 and 7	27.59 ± 2.55	0.55 ± 0.08	672
Probase Cold	1% pH 7	8.70 ± 0.99	0.43 ± 0.05	600
	1% pH 5 and 7	12.83 ± 3.53	0.64 ± 0.13	576
	1% pH 3 and 7	19.43 ± 1.11	0.97 ± 0.13	576
	2.5% pH 7	13.03 ± 2.81	0.26 ± 0.08	576
	2.5% pH 5 and 7	16.85 ± 2.80	0.33 ± 0.07	576
	2.5% 3 and 7	21.35 ± 3.88	0.43 ± 0.11	576
Ufi Gel Hard	1% pH 7	16.66 ± 2.43	0.83 ± 0.21	600
	1% pH 5 and 7	21.00 ± 2.30	0.96 ± 0.09	576
	1% pH 3 and 7	22.08 ± 3.32	1.10 ± 0.10	672
	2.5% pH 7	21.45 ± 1.18	0.43 ± 0.11	672
	2.5% pH 5 and 7	21.92 ± 2.01	0.44 ± 0.15	672
	2.5% pH 3 and 7	24. 88± 2.94	0.59 ± 0.13	672

CHX release was higher at pH 3 and 7, followed by pH 5 and 7, and pH 7, in both CHX 1 and 2.5 % (w/w) incorporation. So, when specimens were subjected to a lower pH, CHX release increased.

As shown in Table 4.1, the greatest amount of CHX release occurred in K (1% and 2.5% at pH 3 and 7), otherwise, PC (CHX 1%) presented the lowest release at pH 7 (8.70 ± 0.99 µg/mL).

At pH 3 and 7, K had a higher release than U, followed by PC. At pH 7 and pH 5 and 7, the pattern of release for K and U seem similar, followed by PC.

In order to compare the three materials at pH 5 and 7, data analysis was applied and showed that the release of CHX from PC was significantly lower compared to both U and K ($p<0.001$) However, it wasn't found significant differences between CHX release from K and U ($p>0.05$).

When pH changes were made, differences between 1 and 2.5% (w/w) specimens were not showed.

4.2. Color Change

After converting CieLch system to CIELab system, Δ**L,** Δ**a** and Δ**b** values were calculated, and Δ**E** was measured. In order to better relate the ΔE to clinical implications according National Bureau of Standards (NBS), the ΔE was transformed by the formula NBS unit = ΔE x 0,92 (Appendix 5).

For all the specimens, a color change was registered.

For each group under study, a descriptive analysis of the data was carried out, to denote if there was difference between pH (Figure 4.8), material (Figure 4.9) and CHX incorporation (Figure 4.10).

Figure 4.10 shows that there were no significant differences in color change between the three released media (p>0.05).

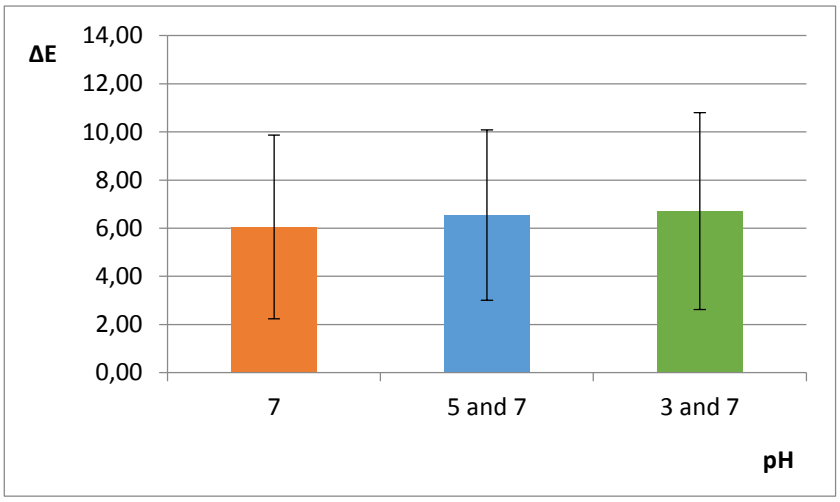


Figure 4.8 – Mean and standard deviation of Δ**E** for each released media.

Kooliner (10.56 ± 2.14) showed the higher color change, followed by Probase Cold (5.72 ± 2.86) and Ufi Gel Hard (3.04 ± 1.21) (p =0.000), as shown in Figure 4.9.

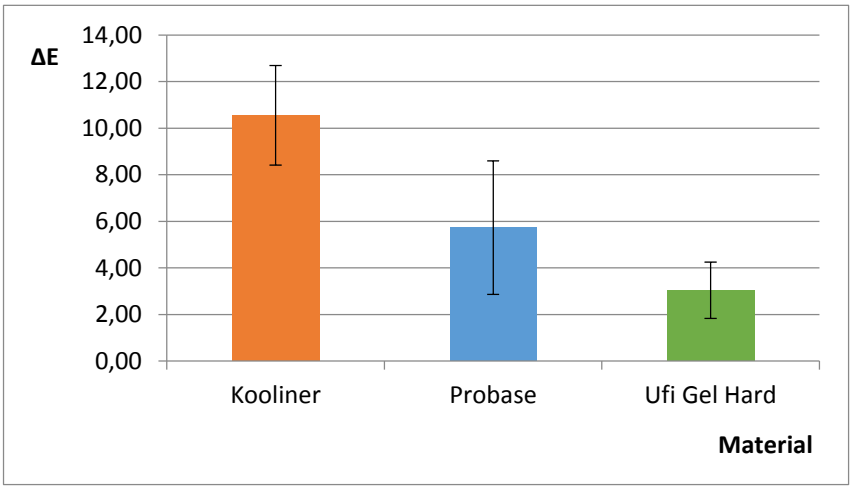


Figure 4.9 – Mean and standard deviation of **ΔE** for each material.

For those specimens without CHX the color change was lower than those with CHX 1 and 2.5% (w/w) (p=0.000). There were no significant differences between CHX 1 and 2.5% (w/w) incorporation (p>0.05) (Figure 4.10).

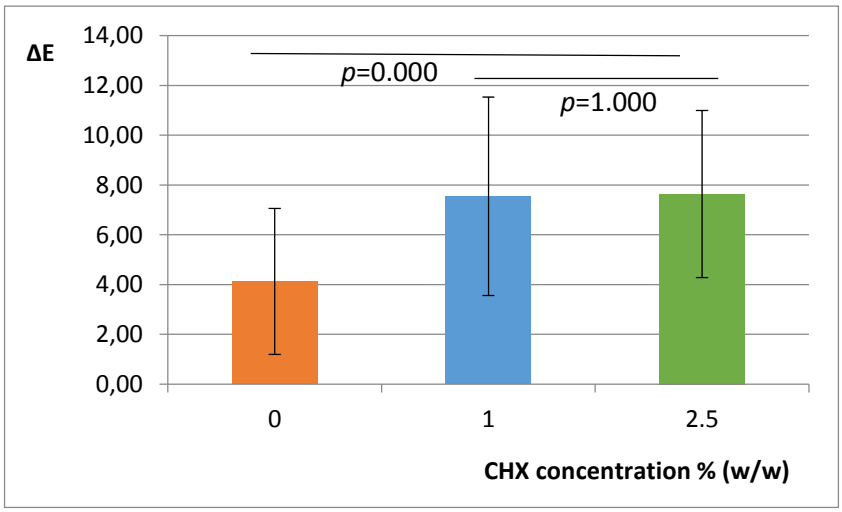


Figure 4.10 – Mean and standard deviation of **ΔE** for each CHX concentration.

5. Discussion

A sustained- release delivery system for treatment of denture stomatitis using chlorhexidine (CHX) incorporated in acrylic reline resins has been suggested to have potential for the prevention of microbial adherence, due to its broad-spectrum antimicrobial activity, including C. *albicans.* (Amin *et al*. 2009; Ryalat *et al*. 2011; Salim, Moore, *et al*. 2012a; Salim, Moore, *et al*. 2013a; Salim, Silikas, *et al.* 2013b).

One of the purposes of the study was to evaluate the effect of pH variation on the release of CHX for each material used.

Several studies use distilled water or artificial saliva at pH 7 as media solution to investigate drug release (Hiraishi *et al*. 2008; Salim, Moore, *et al*. 2012a; Bertolini *et al*. 2014; Marcelino, 2015; Bettencourt *et al*. 2016) but only a few studies test the release in artificial saliva at different pH values (Anusavice *et al*. 2006; Shen *et al.* 2010). It is important to simulate oral cavity conditions, since it is expose to endogenous and exogenous acids (da Mata *et al*. 2009) and, besides that, in case of denture induced stomatitis, the oral cavity pH is lower (pH≈5.2) (Monroy *et al*. 2005).

In this study, the exposure to an acid pH was made in a cyclic way – 6 hours interchanging with 18h at pH 7, because it has been suggested that an individual with a cariogenic diet is subject, daily, to approximately 6 hours of acid environment (Hara *et al*. 2002; da Silva *et al*.2012).

For all the evaluated acrylic reline resins, a high rate of initial release was followed by a slower and steadier release that continued until the end of the study period. This is in agreement with previous studies that associate this change in the rate of drug release with the fact that CHX release is controlled by a concentration dependent diffusion process (Anusavice *et al*. 2006; Gong *et al*. 2007; Hiraishi *et a*l. 2008; Amin *et al*. 2009; Li *et al*. 2009; Ryalat *et al*. 2011; Salim, Moore, *et al*. 2012a; Bertolini *et al*. 2014; Bettencourt *et al*. 2016). The mechanism of release seemed to have two phases: a rapid behavior obeying Fick's law, followed by the formation of fluid clusters around the drug molecules that interact with the mechanism of fluid absorption of the acrylic resins (Riggs *et al*. 2000; Patel *et al*. 2001; Anusavice *et al*. 2006; Amin *et al*. 2009; Ryalat *et al*. 2011).

The results showed that in all the groups studied the greatest amount of CHX release occurred within the first 48h of incubation, as it was observed by Patel *et al*. 2001.

At pH 3 and 7, the maximum cumulative release was higher for the three materials, what can suggest that when the specimens were subjected to a lower pH, there is a higher drug release. This is in agreement with Anusavice *et al.* 2006, who observed that the release of CHX was significantly higher in pH 4 buffer than pH 6, which was attributed to the increase of chlorhexidine diacetate solubility at lower pH. Shen and colleagues in 2010, also concluded that the CHX release increases as the pH of the medium decreased.

Therefore, the first null hypotheses could be rejected, since pH change to an acid condition affect the drug release for all the reline resins.

Both Kooliner (K) and Ufi Gel Had (U) are PEMA based materials known for an anomalous water uptake behaviour (Riggs *et al*. 2000; Patel *et al*. 2001; Salim, Moore, *et al*. 2012a; Salim, Satterthwaite, *et al*. 2012b) that makes them have superior drug release characteristics compared with MMA based materials, as Probase Cold (PC) (Patel *et al*. 2001). In fact, in this study, at pH 3 and 7, K had a higher release than U and at pH 5 and 7 the pattern of release from K and U was similar ($p>0.05$) and PC had a smaller rate of release ($p<0.001$).

Literature suggests that the release of CHX is drug loading-dependent. As the drug loading increases, the pores created by the occupied drug molecules would be larger and/or greater in number and the release of the drug would be higher (Patel *et al*. 2001; Bertolini *et al*. 2014; Marcelino, 2015; Bettencourt *et al*. 2016).

In this study, a 2.5% (w/w) concentration was used to understand if, at a lower pH, it released more than 1% (w/w). For those groups subjected to a pH change there were no significant difference between 1 and 2.5% (w/w) of CHX incorporated. This fact could probably be due to a saturation of the reline resins, after a wide water sorption when pH changes were made. Further studies are needed to better understand this question.

Only a small amount of initial loaded CHX was released to the artificial saliva. This percentage is lower than other studies and it may be due to the smaller dimensions

of the specimens (Patel *et al*. 2001; Salim, Moore, *et al.* 2012a). Even so, the amounts of CHX released appear to be enough.

Since it was not possible to perform microbiological studies, it was assessed a comparison between the cumulative concentrations of CHX and the minimum inhibitory concentration at 24h **(4.05 µg/mL)** and 48h **(5.03 µg/mL)** of incubation, achieved in a study for 32 C. *albicans* isolates (Salim *et al*. 2013a). As it can be observed, all the materials, in all the released media, present a cumulative concentration of CHX superior than C. *albicans* MIC levels, at 24h and 48h.

This is an interesting finding, since it indicates that even CHX 1% could be enough to inhibit C. *albicans*, which encourages the use of CHX in low concentrations, to reduce the risk of developing an allergic reaction by the host, yet possessing an effective antifungal potential (Amin *et al*. 2009; Bertolini *et al*. 2014; Marcelino, 2015). It can also reduce the release of residual monomer (Riggs *et al*. 2000).

In respect of study limitations, it is known that a smaller surface area reduces the drug release by exposing less drug particles to saliva (Salim, Moore, *et al*. 2012a). Despite the specimens used in the study are smaller than the denture surface, the CHX is released from all the cylinder's surfaces, while in a clinical situation only one surface is releasing the drug. So, this can be compensated by the higher number of surfaces releasing CHX. As a high percentage of CHX was not released, other strategies should be considered for the development of local drug acrylic devices, for example, including release enhancers in the material, to avoid the development of multiresistant Candida strains (Bettencourt *et al*. 2016).

To mimic *in vivo* conditions a pH cycling model according a Stefan Curve (da Mata *et al*. 2009) could be considered, in order to have a continuous pH decrease and increased, to simulate saliva buffer capacity. It is essential to standard the experimental protocols in order to compare the results among different studies.

The other purpose of the study was to evaluate the effect of different material composition, CHX incorporation and pH variation in color stability of the three acrylic reline resins.

Color is an important physical property for the aesthetic evaluation of acrylic resins used in the preparation of denture bases.

The *CieLab* system, used in this study, is the most widely used system for measuring instrumental color (Stevenson *et al*. 2010). To avoid reflected light, which practically does not contain color information and may adversely affect the measurement of color, an x-ray chamber was used.

According to previous studies, color differences with corresponding ΔE values lower than 1.0 are not visually detectable by the human eye, and 3.3 NBS (*National Bureau of Standards*) units are acceptable in clinical dentistry (Pero *et al*. 2013; Waldemarin *et al*. 2013; Goiato *et al*. 2014; Moon *et al*. 2015; Sousa *et al*. 2015). In the present study, in all the groups under study, occurred a noticeable color change. Pero *et al*. 2013 also concluded that the incorporation of an antimicrobial polymer into an acrylic resin increased the roughness of surfaces and the wettability, as well as producing color changes with clinical relevance.

These color changes might be related to extrinsic factors that cause discoloration, such as absorption and adsorption of water and to intrinsic factors, like changes in cast or material ageing. When water molecules are absorbed by acrylic resin, they act like plasticizers to damage the material's mechanical resistance through the formation of microcracks related to absorption and hydrolytic degradation of the polymer, resulting in linkage cleavage and gradual deterioration of its infrastructure (Goiato *et al*. 2014).

In regard with pH, there were no differences between groups ($p>0.05$), so, independently of the solution pH there is a color change. Therefore, the second null hypotheses could be accepted.

Kooliner registred the higher color change, followed by Probase Cold and Ufi Gel Hard ($p=0.00$). According to NBS units (Pero *et al.* 2013; Waldemarim *et al*. 2013), K had a *"Much appreciable change"* and PC and U registred a "*Appreciable change".* For U specimens without CHX, ΔE values were clinically acceptable ($1.0 < \Delta E < 3.3$). So, the third null hypotheses could be rejected, since color variation depends on different material composition.

Despite U had a higher release of CHX, it had a smaller color difference, since this material has a tendency to become more apolar (hydrophobic) than Kooliner (Hiraishi *et al.* 2008; Sousa, 2014).

For all the materials, there were differences between those specimens with and without CHX (p=0.00), but there were no differences between CHX 1 and 2.5% (w/w) incorporation (p=1.00). Therefore, the forth null hypotheses could be rejected, since CHX incorporation causes a higher color change. CHX incorporation promotes the formation of micro porosities in the acrylic resin. Its physical presence within the polymer matrix might introduce more spaces and less homogeneity in the polymerized materials. This fact, allied to a higher polarity of the materials, may lead to an increasing water sorption. (Alcantara *et al*. 2012; Salim *et al*. 2012a).

Considering that the clinical relevance of color differences is subjective, future *in vivo* studies could be performed to assess the impact of the color stability of acrylic resins on the satisfaction of patients.

This *in vitro* study simulated a clinical condition in which many other factors can affect color; thus, it has some limitations. It is important to emphasize that other factors, isolated or associated, such as CHX pigmentation (Bevilacqua *et al.* 2016), food and beverages, poor cleaning of prosthesis, components, particles of the oral environment, material porosity associated with the fabrication technique, surface flaws of the material and the polishing surface may influence the color stability of acrylic resins (Goiato *et al*. 2014; Moon *et al*. 2015; Sousa *et al*. 2015). Therefore, additional studies must be performed to evaluate the chemical interactions between coloring agents and acrylic resins.

To resume, a sustained and controlled elution of CHX maintains an effective and gradually increasing concentration of the drug, at the exact site of pathology. Future studies should be carried out with specimens more similar to the denture surfaces and should associate microbiological and biocompatibility tests.

In consideration of drug release and color alteration, Ufi Gel Hard could be an efficient choice for an acute denture candidosis because it will provide higher amounts of CHX released. Then, it can be replaced by Probase Cold, in order to maintain the release and prevent relapses.

Clinical studies are essential, in order to guide the implementation of this systems in clinical practice.

6. Conclusions

Within the limitations of this study, the main conclusions are:

- When subjected to acid conditions, all the evaluated acrylic reline resins had a higher drug release. At a lower pH, the drug release increases.

At pH 5 and 7, the pattern of release of Kooliner (K) and Ufi Gel Hard (U) was similar and Probase Cold (PC) revealed the lowest amounts of chlorhexidine (CHX) released.

- When pH changes were made there was no differences between CHX 1 and 2.5 % (w/w) incorporated.

- pH change, by itself, didn´t affect color stability.

- Different acrylic reline resins composition affects the color stability. K showed the highest color change, followed by PC and U.

- CHX incorporation leaded to a higher color change.

7. References

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Appendices

Appendix 1 – List of Schemes and Tables

Appendix 2 – List of Figures

Appendix 3 – List of Abbreviations

1,6-HDMA	1,6-hexanedioldimethacrylate
C. *albicans*	*Candida albicans*
CHX	Chlorhexidine
h	Hours
IBMA	Isobutylmethacrylate
K	Kooliner
L	Liquid
M	Mean
MIC	Minimum inhibitory concentration
MMA	Methylmethacrylate
P	Powder
PC	Probase Cold
PEMA	Polyethylmethacrylate
PMMA	Polymethylmethacrylate
SD	Standard Deviation
U	Ufi Gel Hard

For Color Measurement

c	Chroma
CIE	Commission International de l'Eclairage
h	Hue
L	Lightness
NBS	National Bureau of Standards
∆E	Overall color change

Appendix 4 – Experimental Data of drug release

1. Kooliner 1% pH 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	2.099	2.040	0.105	0.069
1	3.057	1.040	0.153	0.043
6	4.146	2.243	0.207	0.092
24	6.085	2.018	0.304	0.089
30	6.074	2.170	0.304	0.101
48	11.682	3.398	0.584	0.194
78	7.918	2.627	0.396	0.135
96	9.295	3.467	0.465	0.284
168	11.257	3.350	0.562	0.261
186	11.258	3.230	0.566	0.155
240	10.414	1.352	0.521	0.127
264	12.542	2.110	0.627	0.204
336	12.971	2.705	0.648	0.259
378	13.018	2.774	0.650	0.264
504	14.066	1.540	0.703	0.173
522	14.689	2.544	0.734	0.231
576	16.432	2.146	0.821	0.163
600	13.536	2.674	0.677	0.183
672	14.234	2.590	0.712	0.127

2. Probase Cold 1% pH 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	0.981	0.890	0.0490	0.042
1	3.654	0.395	0.183	0.009
6	4.154	1.978	0.208	0.082
24	4.941	0.873	0.247	0.037
30	8.062	1.335	0.403	0.075
48	8.058	4.648	0.403	0.234
78	3.699	2.077	0.320	0.096
96	7.189	0.494	0.359	0.010
168	7.123	1.522	0.356	0.077
186	6.722	0.692	0.336	0.015
240	8.056	0.633	0.403	0.014
264	8.139	0.959	0.407	0.053
336	8.032	1.100	0.401	0.062
378	8.002	0.943	0.400	0.055
504	8.307	0.554	0.415	0.013
522	8.354	0.692	0.418	0.019
576	8.428	1.454	0.421	0.078
600	8.702	0.986	0.435	0.058
672	8.100	1.434	0.405	0.077

3. Ufi Gel Hard 1% pH 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	4.197	1.814	0.210	0.145
1	4.901	2.619	0.245	0.223
6	7.367	3.559	0.368	0.284
24	8.037	2.852	0.402	0.235
30	8.266	1.081	0.413	0.044
48	11.115	2.041	0.556	0.183
78	11.517	2.943	0.576	0.244
96	10.291	1.523	0.514	0.132
168	10.776	3.046	0.539	0.256
186	12.055	2.307	0.603	0.213
240	14.312	0.696	0.715	0.009
264	14.197	3.735	0.710	0.297
336	14.456	2.905	0.723	0.264
378	15.066	2.551	0.753	0.219
504	14.603	1.709	0.730	0.143
522	14.489	0.816	0.724	0.012
576	16.391	2.818	0.819	0.236
600	16.666	2.433	0.833	0.214
672	15.204	0.402	0.760	0.005

4. Kooliner 1% pH 5 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	2.289	1.542	0.141	0.021
1	4.747	2.502	0.237	0.036
6	5.675	3.039	0.284	0.057
24	7.776	2.863	0.389	0.042
30	8.340	6.126	0.417	0.195
48	12.850	5.388	0.642	0.072
78	13.896	1.197	0.695	0.027
96	13.370	3.173	0.668	0.074
168	14.823	1.065	0.741	0.035
186	12.034	5.940	0.602	0.174
240	18.181	4.176	0.909	0.102
264	21.928	1.142	1.096	0.039
336	15.939	2.478	0.797	0.094
378	22.123	3.257	1.106	0.082
504	19.393	2.297	0.969	0.165
522	22.145	2.134	1.107	0.104
576	21.277	1.875	1.064	0.037
600	20.79	1.924	1.039	0.039
672	20.11	2.104	1.005	0.054

5. Probase Cold 1% pH 5 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	1.019	0.687	0.051	0.003
1	1.286	1.051	0.064	0.038
6	2.035	0.811	0.102	0.006
24	4.839	3.276	0.242	0.129
30	3.961	2.202	0.198	0.083
48	5.148	2.621	0.257	0.094
78	4.370	1.212	0.218	0.048
96	7.887	2.522	0.394	0.092
168	6.823	3.309	0.341	0.131
186	6.210	0.970	0.310	0.006
240	9.594	3.050	0.480	0.127
264	7.866	1.649	0.393	0.068
336	9.298	1.612	0.465	0.066
378	9.078	1.859	0.454	0.084
504	12.359	1.898	0.618	0.086
522	11.778	3.044	0.589	0.117
576	12.828	3.535	0.641	0.138
600	11.158	0.970	0.558	0.008
672	10.903	1.247	0.545	0.072

6. Ufi Gel Hard 1% pH 5 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0.236	0
0.33	4.713	0.966	0.253	0.008
1	5.060	3.462	0.357	0.221
6	7.151	3.361	0.396	0.218
24	7.924	0.163	0.487	0.002
30	9.735	5.378	0.503	0.413
48	10.065	2.244	0.574	0.108
78	11.478	4.802	0.678	0.325
96	13.562	3.171	0.631	0.212
168	12.632	1.360	0.712	0.053
186	14.244	2.127	0.816	0.087
240	16.320	5.012	0.843	0.402
264	16.872	1.116	0.887	0.048
336	17.736	2.586	0.934	0.114
378	18.685	3.601	0.872	0.246
504	17.436	3.151	0.972	0.206
522	19.447	3.504	1.050	0.241
576	21.001	2.296	1.050	0.094
600	18.745	1.252	0.937	0.052
672	20.526	2.448	1.026	0.106

7. Kooliner 1% pH 3 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	4.161	3.242	0.208	0.092
1	2.586	1.033	0.129	0.047
6	10.148	4.086	0.507	0.106
24	7.710	3.644	0.385	0.094
30	12.200	1.105	0.610	0.049
48	12.004	2.356	0.600	0.086
78	16.822	4.957	0.841	0.174
96	17.537	4.203	0.877	0.123
168	24.306	4.480	1.215	0.146
186	24.468	4.931	1.223	0.168
240	23.357	3.584	1.168	0.093
264	20.323	0.593	1.016	0.007
336	19.474	0.487	0.973	0.006
378	24.016	3.528	1.201	0.089
504	26.996	2.874	1.350	0.085
522	27.243	1.545	1.362	0.074
576	25.852	1.987	1.292	0.080
600	29.609	1.665	1.480	0.079
672	25.199	2.836	1.260	0.081

8. Probase Cold 1% pH 3 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	3.588	1.780	0.179	0.163
1	4.044	2.423	0.202	0.198
6	4.455	1.830	0.223	0.171
24	7.517	3.349	0.376	0.243
30	5.490	2.025	0.274	0.178
48	10.586	4.358	0.529	0.208
78	11.178	2.274	0.559	0.195
96	10.148	2.191	0.507	0.189
168	13.001	1.533	0.650	0.148
186	12.840	1.840	0.331	0.172
240	14.353	3.031	0.717	0.213
264	11.906	1.333	0.595	0.134
336	13.894	2.141	0.695	0.176
378	12.052	0.323	0.602	0.084
504	18.818	3.349	0.941	0.245
522	16.777	2.685	0.839	0.203
576	19.430	1.108	0.971	0.132
600	16.392	0.975	0.819	0.102
672	19.076	2.077	0.954	0.186

9. Ufi Gel Hard 1% pH 3 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	3.655	2.816	0.183	0.107
1	5.254	3.372	0.263	0.114
6	5.080	2.028	0.254	0.083
24	6.998	3.209	0.350	0.107
30	7.900	4.212	0.394	0.216
48	9.548	3.508	0.477	0.128
78	11.446	4.544	0.572	0.264
96	12.653	3.636	0.632	0.129
168	12.179	4.134	0.609	0.203
186	13.746	5.252	0.687	0.298
240	16.164	4.773	0.808	0.275
264	16.581	4.142	0.829	0.206
336	17.922	1.896	0.896	0.058
378	17.486	1.903	0.874	0.063
504	20.344	1.884	1.017	0.057
522	19.729	3.540	0.986	0.126
576	21.251	2.482	1.062	0.097
600	21.101	2.072	1.055	0.089
672	22.078	3.320	1.104	0.102

10. Kooliner 2.5% pH 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	3.445	0.299	0.069	0.006
1	5.667	1.741	0.113	0.085
6	7.180	3.765	0.143	0.147
24	8.449	4.224	0.169	0.243
30	7.724	2.765	0.154	0.122
48	14.775	3.359	0.295	0.136
78	14.502	2.448	0.290	0.109
96	14.536	3.361	0.291	0.138
168	15.930	3.565	0.318	0.146
186	16.831	4.189	0.336	0.234
240	17.362	2.587	0.347	0.118
264	17.450	1.554	0.349	0.074
336	19.731	2.750	0.350	0.132
378	17.522	2.422	0.360	0.106
504	17.997	1.182	0.393	0.054
522	19.669	1.523	0.426	0.072
576	21.330	1.740	0.429	0.083
600	21.477	1.413	0.395	0.067
672	19.768	1.872	0.427	0.094

11. Probase Cold 2.5% pH 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	2.467	1.764	0.049	0.032
1	4.369	2.002	0.087	0.053
6	3.774	2.307	0.075	0.042
24	6.338	4.375	0.127	0.086
30	7.499	4.566	0.150	0.098
48	10.103	3.545	0.202	0.078
78	7.957	2.614	0.159	0.065
96	6.980	0.643	0.139	0.012
168	9.012	2.252	0.180	0.052
186	8.711	2.353	0.175	0.061
240	9.984	2.070	0.200	0.041
264	10.539	1.823	0.211	0.035
336	10.577	2.114	0.211	0.045
378	11.295	2.077	0.225	0.042
504	12.438	2.620	0.249	0.068
522	11.953	1.816	0.239	0.034
576	13.028	2.809	0.260	0.076
600	12.729	1.625	0.254	0.021
672	11.470	1.263	0.229	0.018

12. Ufi Gel Hard 2.5% pH 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	4.556	3.915	0.091	0.067
1	4.607	0.816	0.092	0.007
6	10.420	4.248	0.208	0.109
24	9.722	1.680	0.194	0.099
30	9.72	0.707	0.194	0.006
48	12.77	3.862	0.255	0.059
78	13.661	3.933	0.273	0.082
96	13.327	4.146	0.266	0.111
168	12.920	2.012	0.258	0.085
186	14.057	3.402	0.281	0.097
240	17.412	3.753	0.348	0.099
264	16.922	3.992	0.338	0.124
336	17.845	2.828	0.357	0.093
378	18.746	2.833	0.375	0.096
504	19.455	2.179	0.389	0.068
522	18.801	0.626	0.376	0.014
576	20.697	0.718	0.414	0.016
600	20.793	1.731	0.416	0.103
672	21.453	2.177	0.429	0.109

13. Kooliner 2.5% pH 5 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	1.333	1.692	0.027	0.004
1	1.757	1.446	0.035	0.008
6	5.152	3.242	0.103	0.085
24	5.372	2.113	0.107	0.071
30	7.622	3.001	0.152	0.132
48	11.976	5.799	0.239	0.202
78	12.586	3.865	0.252	0.154
96	12.169	4.732	0.243	0.184
168	12.637	2.545	0.253	0.113
186	15.881	3.694	0.317	0.152
240	14.495	3.426	0.290	0.137
264	15.035	3.694	0.301	0.151
336	18.786	2.992	0.375	0,127
378	18.601	5.645	0.368	0.304
504	18.394	0.700	0.342	0.034
522	22.124	3.013	0.442	0.132
576	20.104	3.884	0.402	0.154
600	18.776	0.816	0.375	0.032
672	20.674	0.906	0.413	0.045

14. Probase Cold 2.5% pH 5 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	1.448	0.764	0.029	0.004
1	1.613	0.665	0.032	0.002
6	3.989	1.212	0.080	0.032
24	5.109	3.723	0.102	0.098
30	4.320	2.101	0.086	0.042
48	7.412	4.556	0.148	0.102
78	6.078	0.404	0.121	0.001
96	10.673	4.568	0.213	0.143
168	9.991	2.427	0.200	0.087
186	8.991	1.131	0.180	0.074
240	12.733	2.600	0.255	0.089
264	11.983	3.501	0.240	0.102
336	13.503	2.618	0.270	0.083
378	14.609	1.542	0.292	0.072
504	15.988	3.854	0.320	0.179
522	13.871	0.808	0.277	0.039
576	16.851	3.805	0.337	0.174
600	16.540	2.344	0.331	0.142
672	14.282	2.536	0.286	0.151

15. Ufi Gel Hard 2.5% pH 5 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	4.704	4.202	0.094	0.071
1	4.122	1.733	0.082	0.013
6	6.228	3.753	0.124	0.064
24	7.663	3.162	0.153	0.078
30	9.509	0.425	0.190	0.014
48	11.082	4.860	0.222	0.108
78	11.933	2.392	0.239	0.064
96	13.882	2.393	0.278	0.067
168	14.414	4.180	0.288	0.176
186	14.676	2.896	0.293	0.128
240	16.855	2.890	0.337	0.127
264	17.472	2.470	0.349	0.121
336	18.193	0.753	0.364	0.016
378	20.255	0.951	0.405	0.017
504	19.902	3.767	0.398	0.189
522	21.646	1.506	0.433	0.087
576	21.414	1.795	0.428	0.098
600	21.445	2.561	0.429	0.152
672	21.921	2.012	0.438	0.104

16. Kooliner 2.5% pH 3 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	7.484	2.824	0.150	0.065
1	5.148	1.501	0.103	0.053
6	11.439	3.383	0.229	0.072
24	9.667	2.066	0.193	0.061
30	13.006	1.176	0.260	0.055
48	10.743	2.362	0.215	0.063
78	18.195	3.983	0.364	0.092
96	17.189	4.044	0.344	0.114
168	19.466	4.745	0.389	0.126
186	18.269	4.824	0.365	0.131
240	23.914	4.815	0.478	0.129
264	18.825	0.898	0.376	0.034
336	23.548	2.413	0.471	0.105
378	27.481	1.945	0.549	0.112
504	24.243	2.154	0.484	0.107
522	27.436	2.546	0.549	0.092
576	25.942	1.986	0.519	0.087
600	26.690	1.793	0.534	0.085
672	27.587	2.549	0.551	0.083

17. Probase Cold 2.5% pH 3 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	1.158	0.986	0.023	0.012
1	4.564	4.296	0.091	0.038
6	4.854	3.589	0.097	0.029
24	7.003	0.989	0.140	0.009
30	4.566	1.749	0.091	0.034
48	12.321	2.358	0.246	0.053
78	9.885	4.307	0.198	0.102
96	13.221	3.712	0.264	0.085
168	14.208	2.781	0.284	0.064
186	11.785	1.941	0.236	0.038
240	15.616	4.189	0.312	0.101
264	13.300	2.084	0.266	0.057
336	15.038	1.646	0.301	0.078
378	15.607	1.947	0.312	0.085
504	20.903	2.915	0.418	0.098
522	20.333	0.781	0.407	0.014
576	21.355	3.880	0.427	0.114
600	18.135	0.841	0.363	0.028
672	21.053	2.892	0.421	0.096

18. Ufi Gel Hard 2.5% pH 3 and 7

Time Intervals (hours)	M (cumulative concentration)	SD (cumulative concentration)	M (% released)	SD (% released)
0	0	0	0	0
0.33	4.988	2.675	0.100	0.047
1	5.598	0.816	0.112	0.011
6	4.769	1.764	0.095	0.018
24	8.806	3.676	0.176	0.052
30	11.577	0.707	0.231	0.017
48	12.452	3.979	0.249	0.104
78	13.866	3.475	0.277	0.098
96	15.380	2.620	0.307	0.073
168	15.184	4.128	0.304	0.126
186	15.614	3.086	0.312	0.111
240	17.412	2.677	0.348	0.103
264	17.674	1.865	0.353	0.095
336	19.708	4.332	0.394	0.133
378	19.968	0.835	0.399	0.045
504	20.433	3.254	0.409	0.137
522	23.424	3.612	0.468	0.142
576	23.938	1.054	0.479	0.059
600	23.440	1.848	0.469	0.082
672	24.880	2.939	0.497	0.134

Appendix 5 – Experimental Data of Color Measurement

1. Mean (M) and standard deviation (SD) values of ΔE and NBS classification.

Material	**CHX Concentration % (w/w)**	**pH**	**ΔE**		**NBS**
			M	SD	
Kooliner	0 (Control Group)	7	8.92	0.52	8.21
		5 and 7	7.88	0.41	7.25
		3 and 7	7.51	0.96	6.91
	1	7	11.42	1.40	10.51
		5 and 7	11.75	0.69	10.81
		3 and 7	13,19	1.11	12.13
	2.5	7	11.06	0.84	10.18
		5 and 7	10.34	1.18	9.51
		3 and 7	12.94	0.59	11.90
Probase Cold	0 (Control Group)	7	1.89	0.57	1.74
		5 and 7	1.96	0.38	1.80
		3 and 7	2.01	0.48	1.85
	1	7	7.16	1.20	6.58
		5 and 7	8.13	1.28	7.48
		3 and 7	7.72	0.30	7.10
	2.5	7	6.54	1.53	6.01
		5 and 7	8.65	0.47	7.96
		3 and 7	7.47	0.47	6.88
Ufi Gel Hard	0 (Control Group)	7	1.84	0.52	1.70
		5 and 7	2.27	0.65	2.09
		3 and 7	2.89	0.80	2.66
	1	7	2.31	1.02	2.12
		5 and 7	3.32	1.32	3.05
		3 and 7	2.91	0.66	2.68
	2.5	7	3.34	1.13	3.08
		5 and 7	4.67	1.40	4.29
		3 and 7	3.78	0.97	3.48